

## Review

# Calorie restriction and the nutrient sensing signaling pathways

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**Abstract.** Calorie restriction (CR) is the most potent regimen known to extend the life span in multiple species. CR has also been shown to ameliorate several age-associated disorders in mammals and perhaps humans. CR induces diverse metabolic changes in organisms, and it is currently unclear whether and how these metabolic changes lead to life span extension. Recent studies in model systems have provided insight

into the molecular mechanisms by which CR extends life span. In this review, we summarize and provide recent updates on multiple nutrient signaling pathways that have been connected to CR and longevity regulation. The roles of highly conserved longevity regulators – the Sirtuin family – in CR are also discussed.

**Keywords.** Calorie restriction, aging, Sir2, TOR, insulin/PI3K, Akt/PKB, mitochondria.

## Introduction

Aging is a complex process associated with gradual loss of physiological functions, regulated by genetic and environmental factors. Recent studies in genetically tractable model systems including yeast, worms, flies and mice demonstrate that longevity can be modulated by single gene mutations [1–9]. Manipulations of these longevity genes not only extend life span but also delay many age-associated phenotypes, suggesting aging is a regulated process and longevity assurance genes govern the rate of aging [2, 7–9].

Calorie restriction (CR, a moderate reduction in calorie intake), also called dietary restriction (DR), is the most effective intervention known to extend life span in a variety of species including mammals (reviewed in [10, 11]). CR has also been shown to delay the onset or reduce the incidence of many age-related diseases including cancer, diabetes and car-

diovascular disorders [10–12]. For example, CR suppresses the carcinogenic effect of several classes of chemicals and several forms of radiation-induced cancers in rodents [13–15]. CR also inhibits a variety of spontaneous neoplasias in experimental model systems, including tumors arising in several knockout and transgenic mouse models such as p53-deficient mice [13]. Recent studies suggest that CR may also induce beneficial effects in primates. CR in primates evokes beneficial changes in biomarkers, such as insulin sensitivity, body weight, temperature and blood pressure, and quantity and type of lipids [11, 16, 17]. Although it is unclear whether CR has the same effects on humans, CR appears to improve several markers of aging such as the levels of blood glucose, blood pressure and cholesterol [17–19].

CR may work by reducing the levels of reactive oxygen species (ROS) due to a slower metabolism [10, 11]; however, the mechanism by which CR extends longevity and ameliorates age-associated diseases remains unclear. Recent studies have put forward several hypotheses to help clarify the mechanisms of

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CR (reviewed in [12, 20, 21]). In this review we summarize recent findings on the conserved nutrient sensing pathways that extend life span in multiple species with a primary focus on studies in the unicellular model organism-budding yeast *Saccharomyces cerevisiae*, and a secondary focus on their counterparts in higher eukaryotes.

### Overview – Yeast as a model system for studying longevity regulation

The budding yeast *S. cerevisiae* provides an efficient model for exploring the molecular mechanisms of longevity regulation. Owing to the short life cycle and well-established molecular genetic techniques in this system, yeast mutants and the corresponding genes that alter life span are relatively easy to obtain. In addition, recent studies have shown that several longevity factors and pathways are highly conserved [7]. Therefore, this unicellular eukaryote represents a powerful system to identify new components in the longevity regulating pathways and to study these pathways at the molecular/genetic level.

Yeast life span has been measured and studied in two distinct ways: Replicative life span (division potential) and chronological life span (cell survival at a non-dividing state). Several longevity factors/pathways have been independently identified through both types of studies. However, the extent of overlap between the different aspects of life span regulation measured by these two types of studies is still unclear. The replicative life span is characterized by the number of divisions an individual cell undergoes before senescence [22–24]. Cell division in *S. cerevisiae* is asymmetrical, the replicative life span of mother cells can thus be accurately measured by moving small daughters away from the mothers via microscopic manipulation. Yeast mother cells stop dividing after an average of ~22 divisions, depending on strain background and culture conditions. Cultured mammalian cells undergo a limited number of divisions before entering a state of replicative senescence [25–28]. While the senescence of human cells is mainly determined by their telomere length or structure, other factors influence cellular senescence as well [27, 28]. Although the extent of overlap between mammalian cellular senescence and yeast aging is presently unclear, certain signaling proteins, such as sirtuins and TOR (target of rapamycin) network components have been shown to regulate both yeast replicative life span (as discussed in this review) [29, 30] and mammalian cellular senescence [28, 31]. Although many studies have established a link between the longevity *in vivo* and the proliferative potential of mammalian cells in

culture [32, 33], significant correlation between replicative potential of human cells and donor age has not been reliably demonstrated [28, 34]. While replicative senescence can have both beneficial and detrimental effects on organismal longevity, it is conceivable that certain genetic or environmental factors that regulate cellular replicative potential affect organismal aging as well [27, 35]. Insulin/IGF-1 and p53 signaling pathways are examples of genetic factors influencing aging at both cellular and organismal levels [28, 35, 36].

Chronological life span refers to the length of time cells remain viable in a non-proliferating state (stationary phase or post-diauxic phase) [37]. Stationary phase yeast cells exhibit a number of phenotypes reminiscent of the G<sub>0</sub> state (post-mitotic) of higher eukaryotic cells [38]. It has also been shown that yeast stationary phase cells undergo apoptosis after prolonged culture [39, 40]. Therefore, it is conceivable that there is a potential commonality between longevity mechanisms operating in quiescent yeast cells and post-mitotic mammalian cells. For example, increased stress resistance can be beneficial for both unicellular yeast and multicellular organisms by protecting their irreplaceable non-dividing cells from age-associated damage [37, 41]. Owing to the evolutionary conservation in longevity regulating factors, as described in this and other articles, studies of yeast replicative and chronological life spans can enrich our understanding of longevity regulation in multicellular organisms.

Several signaling pathways have been implicated in yeast longevity regulation. (Most yeast studies discussed in this review refer to replicative life span unless otherwise indicated.) Decreasing the activity of the glucose sensing cyclic AMP (cAMP)-dependent protein kinase A pathway (PKA) (which regulates cell proliferation and stress response) extends life span [5, 42]. The *tor1Δ* and *sch9Δ* mutants, have recently been reported to extend yeast life span [6, 30]. The nutrient-sensing TOR pathway and Sch9 kinase (a homolog of the Akt kinase family) are known to interact with the PKA pathway to regulate cell growth [43, 44]. The Snf1 kinase (the mammalian AMP kinase homolog in yeast) complex, which functions to derepress glucose-repressed genes (required for alternative carbon source utilization) has also been shown to regulate life span [4, 45, 46]. Furthermore, the retrograde signaling pathway (mediated by the Rtg proteins) that senses mitochondrial function to influence the transcription of nuclear genes plays an important role in longevity [47]. Here we summarize these pathways in further detail and discuss how these pathways interact with CR to regulate longevity.

## CR and the glucose responsive pathways in yeast and in multicellular eukaryotes

### CR and the glucose-sensing Ras-cAMP/PKA pathway in yeast

Moderate CR can be imposed in the budding yeast *S. cerevisiae* by reducing the glucose concentration from 2% to 0.5% in rich media [5, 48–52]. Under this CR condition, the growth rate remains robust and yeast mother cells show an extended replicative life span of about 20–30%. Variations in CR protocols have been described where limitation of amino acids and other nutrients accompany carbon source reduction [53, 54]. These regimens may impact on other longevity pathways that function in parallel to CR in rich media.

One of the first signaling pathways implicated in regulation of longevity in yeast is the Ras-cAMP/PKA pathway (Fig. 1a) [5, 6, 42] that links glucose availability with the control of growth, proliferation, metabolism, stress resistance, and longevity [55, 56]. Multiple signals are required for the activation of the PKA pathway: the signal for glucose availability transduced through the G-protein-coupled receptor Gpr1 and the G $\alpha$  protein Gpa2, and a glucose phosphorylation event by glucokinase (Glk1) or hexokinase (Hxk1 or 2) are required for adenylate cyclase Cyr1 (Cdc35) activation. Upon stimulation by the GTP/GDP binding Ras proteins (Ras1, Ras2), Cyr1 produces cAMP, which activates PKA by promoting the dissociation of the regulatory subunits (Bcy1) from the catalytic subunits Tpk1, 2 and 3 [55, 57, 58]. Mutations in components of this pathway extend yeast life span in multiple strain backgrounds and are recognized as genetic models of CR [5, 48, 50–52, 59, 60]. These CR genetic models include the hexokinase mutant (*hxx24*) and mutations that down-regulate the glucose-sensing cAMP/PKA pathway: the temperature-sensitive alleles of the adenylate cyclase (*cdc35-1*) or the RAS GTP/GDP exchange protein (*cdc25-10*) and deletions of the glucose-sensing protein Gpa2 and Gpr1. In addition, mutations in Cyr1 or Ras2 also confer chronological life span extension (Fig. 1b) [6, 37].

### The yeast Sch9 pathway

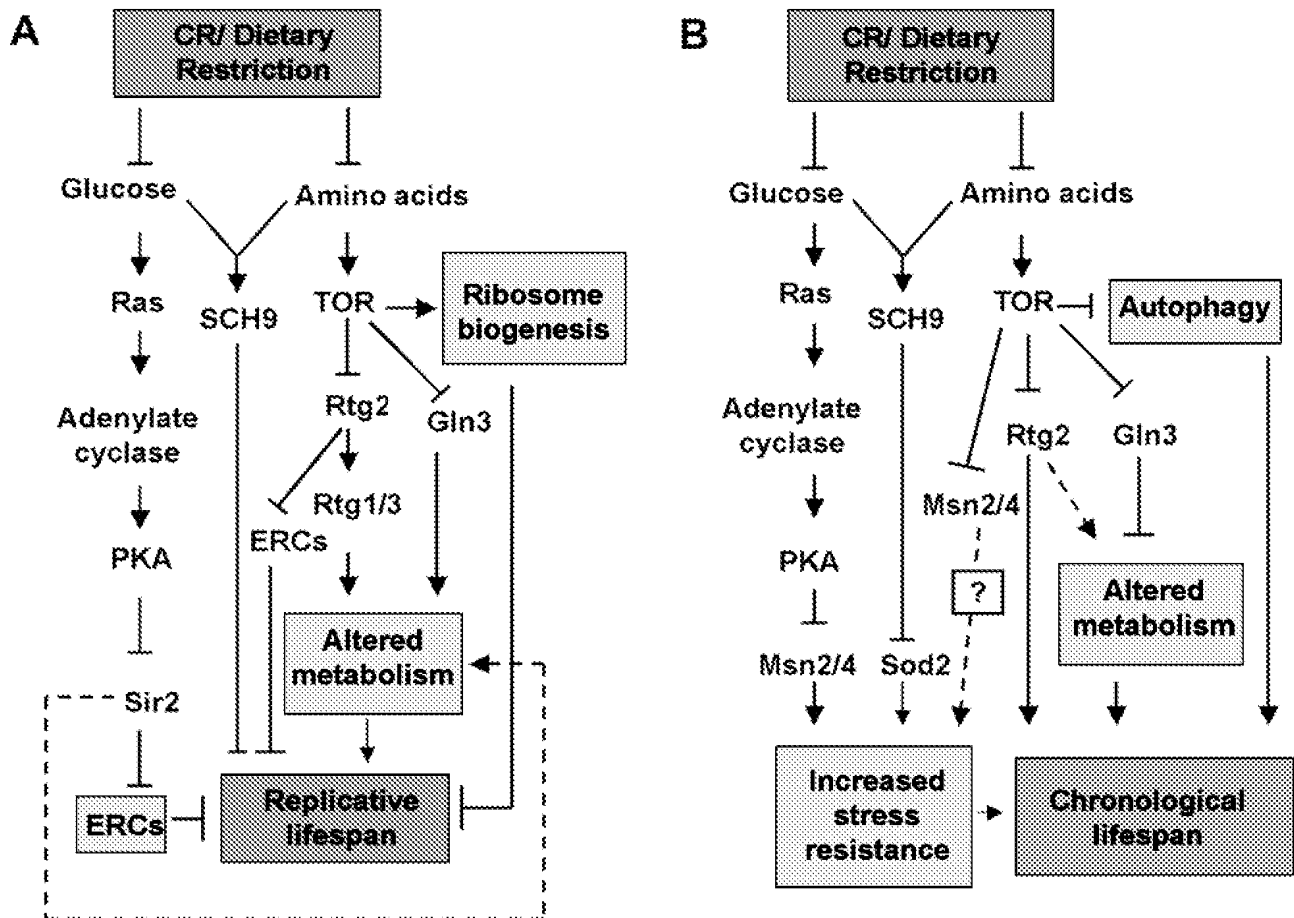
Sch9 is a serine/threonine protein kinase with homology to yeast PKA and mammalian protein kinase B (PKB/Akt) in the catalytic subunit [61, 62]. In addition to longevity regulation, Sch9 is involved in the control of cell size and oxidative stress resistance [6, 63, 64]. Sch9 is required for the response of nitrogen (N)-starved glucose-repressed cells to the re-addition of N source [65]. Thus, Sch9 is a central component of the “fermentable growth medium” (FGM)-induced pathway, which monitors the pres-

ence of fermentable sugar (such as glucose), and all essential nutrients, (such as N source) in the growth media [65, 66]. PKA and Sch9 appear to function in partially overlapping pathways [65, 66]. Sch9 over-expression suppresses the lethality of the triple deletions of the *TPK1-3* genes (PKA catalytic subunits) [61]. Conversely, enhanced PKA activity suppresses the decrease in proliferation rate caused by deleting *SCH9* [61]. A recent genome-wide expression study confirms that Sch9 and PKA indeed share many common targets [67]. The *sch9 $\Delta$*  mutant extends both replicative and chronological life span in yeast (Fig. 1a, b) [6, 30]. It has been suggested that Sch9 acts in the CR pathway to extend replicative life span [30].

### Insulin signaling in multicellular eukaryotes

The Sch9 homolog, PKB/Akt, also regulates life span as part of the insulin/phosphatidylinositol 3 kinase (PI3K) signaling pathway, which has a major impact on aging in multicellular organisms [36, 68–73]. Upon binding of insulin/insulin-like growth factor 1 (IGF-1) to the receptor, PI3K is recruited and activated, creating second messengers like phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P<sub>3</sub>) and phosphatidylinositol-3,4-bisphosphate (PI-3,4-P<sub>2</sub>), which ultimately recruit and stimulate a number of downstream kinases such as PKB [68, 72].

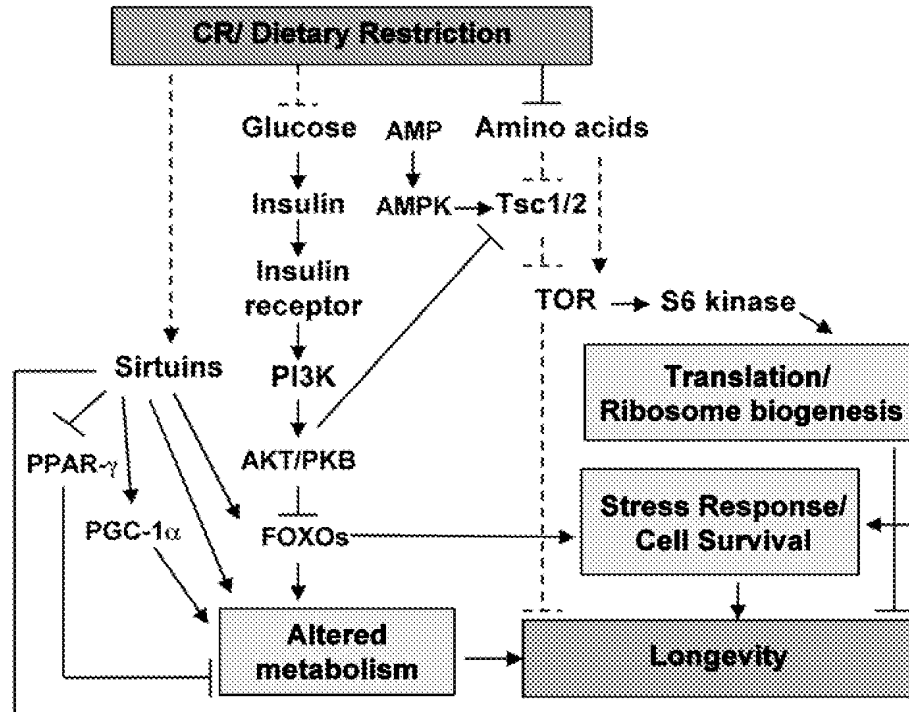
Decreased signaling through the insulin/IGF-1 pathway increases life span in worms, flies and mice (Fig. 2) [74–79]. Flies (*Drosophila melanogaster*) carrying mutations in the insulin-like receptor (*InR*) are sterile dwarfs with increased life span in females [76]. Similarly, female heterozygous IGF-1 receptor knockout mice live 33% longer than same sex wild-type mice but do not display any growth or behavioral alterations [79]. Mice lacking the insulin receptor, specifically in adipose tissue, also exhibit longer life span and do not display any growth defects [78]. In worms (*Caenorhabditis elegans*), life span extension induced by mutations in the insulin/IGF-1 receptor DAF-2 and in the PI3K, AGE-1, requires the conserved forkhead transcription factor, DAF-16 (a mammalian FOXO homolog) [36, 71, 73, 80, 81]. The role of FOXO transcription factors in extending longevity is likely to be conserved, since increased expression of dFOXO in the fly head fat body also increases longevity [82]. Although CR reduces insulin and IGF-1 levels in animals [18, 69], it remains highly debatable whether CR functions through insulin/IGF-1 signaling to extend life span. Studies in worms indicate that CR does not extend life span through the insulin/IGF-1 pathway [83–85]. However, one common feature between CR and reduction in signaling through yeast Ras-cAMP/PKA and Sch9, TOR, as well as multicellular insulin/IGF-1 pathways, is the decrease



**Figure 1.** Calorie restriction (CR) and nutrient-sensing longevity-regulating pathways in yeast. Reducing signaling through Ras/PKA, Sch9 and TOR pathways extends (a) replicative and (b) chronological life span in budding yeast. Extension of replicative life span by CR (reduced glucose concentration in growth media from 2% to 0.5%) or mutations decreasing PKA activity requires the NAD-dependent histone deacetylase Sir2. Sir2 inhibits the formation of extrachromosomal ribosomal DNA circles (ERCs), which is one of the factors causing yeast aging. Sir2 may also regulate other downstream targets such as metabolic enzymes to extend life span. Chronological life span extension by Ras/PKA pathway mutants is abolished by deletions of the stress response transcription factors Msn2 and Msn4. Although increased chronological life span in the *sch9Δ* mutant is abrogated by deleting *SOD2* (encoding a mitochondrial superoxide dismutase), the mechanism by which *sch9Δ* extends replicative life span is still unknown. Disruption of TOR signaling increases replicative (a) and chronological (b) life span through modulation of a number of downstream TOR effectors and transcriptional targets. In particular, replicative life span was extended by deletions of ribosomal proteins or the inhibitor of the transcription factor Gln3, involved in the uptake and metabolism of alternative nitrogen sources. Conversely, the *gln3Δ* mutation extends chronological life span and this life span extension is decreased by deletions of the PKA and TOR downstream effectors, Msn2 and Msn4. TOR-controlled autophagy also affects chronological life span. Additional TOR effectors, Rtg1, Rtg2, and Rtg3, which are central players in the retrograde response, also modulate yeast longevity. Rtg2 is suggested to extend replicative life span by suppressing ERC formation (at least in part); however, the mechanism by which Rtg2 influences chronological life span remains unknown. Although not shown in this figure, Snf1, the yeast homologue of mammalian AMP-activated kinase (AMPK), has also been implicated in regulation of yeast longevity. Arrows designate activation, bars designate inhibition, and the dotted lines signify a suggested functional relationship. For clarity, not all functional relationships are shown.

in glucose metabolism [55, 67, 69, 72, 86–88]. Two groups studying *C. elegans* have reported that reduction of glycolysis through interfering RNA (RNAi) treatments targeting the glycolysis enzymes phosphoglycerate mutase and glucose-6-phosphate isomerase extend worm life span in a DAF-16-dependent manner [89, 90], thus linking the insulin/IGF-1 pathway with known CR-mediated changes in metabolism. Furthermore, the insulin/IGF-1 pathway appears to mediate CR effects in flies and mice [85, 91, 92]. In

flies, CR does not further extend the life span of long-lived *chico* (encodes an insulin receptor substrate) mutants [85, 91]. Similarly, CR does not further extend the life span of long-lived growth hormone (GH) receptor/GH-binding protein knockout (GHRKO) mice [92]. Identification and characterization of new components in both CR and the insulin/IGF-1 pathways will help elucidate whether these two pathways indeed function by similar mechanisms.



**Figure 2.** CR and nutrient-sensing longevity regulating pathways in multicellular eukaryotes. TOR and insulin/PI3K pathways are two major nutrient-sensing pathways that modulate longevity in metazoans. Reducing insulin/PI3K signaling through mutations in the insulin/insulin-like growth factor 1 (IGF-1) receptor(s), phosphatidylinositol-3-kinase (PI3K), or Akt/protein kinase B (PKB) extends life span in worms, flies and rodents. One primary downstream effector of the insulin/PI3K pathways in multicellular organisms is the FOXO transcription factor family that controls stress response/cell survival and metabolism. In worms, life span extension by overexpressing sirtuins is dependent on FOXO-like transcription factor (DAF-16). In mammals, sirtuins appear to influence cell survival and metabolism through both FOXO-dependent and FOXO-independent mechanisms. For example, sirtuins regulate fat and glucose metabolism through peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) and PPAR- $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ), respectively. In flies, one TOR downstream effector S6 kinase has been shown to modulate longevity. It is currently unknown whether TOR signaling regulates life span in mammals. In flies and mammals, insulin/PI3K signaling to TOR is mediated by Tsc1/Tsc2, which are absent in worms [209]. While worm AAK-2 (AMP-activated kinase) has been shown to extend life span, it is not known whether it functions upstream of TOR as in mammalian cells. Although not shown in this figure, it has been demonstrated that life span extension by a loss-of-function mutation in the insulin-like receptor in worms requires the autophagy protein BEC-1 [210]. Arrows designate activation, bars designate inhibition, and the dotted lines signify a suggested functional relationship. For clarity, not all functional relationships are shown.

### TOR signaling pathway

The TOR signaling pathway is another nutrient responsive pathway recently found to be important in longevity regulation (Figs 1 and 2) [30, 93–98]. The TOR signaling network plays a central role in regulating growth of proliferating and non-proliferating eukaryotic cells in response to nutritional signals, such as nitrogen and carbon sources [99, 100]. TOR controls cell growth and proliferation through impinging on a diverse array of cellular processes at the level of transcription, translation and intracellular trafficking [99, 100]. TOR is a highly conserved serine/threonine protein kinase belonging to the phosphatidylinositol kinase-related kinase family (PIKK) [99, 100]. TOR is also the specific cellular target of rapamycin, an immunosuppressant and antiproliferative drug [99]. Prolonged treatment with rapamycin or TOR deletion in yeast causes phenotypic changes characteristic of starved ( $G_0$ ) cells: altered transcrip-

tion pattern, down-regulation of protein synthesis, accumulation of storage carbohydrates and acquisition of thermo-tolerance [101]. There are two TOR genes in budding yeast, *TOR1* and *TOR2*. Although *TOR1* and *TOR2* are highly homologous, only *TOR2* is essential for growth [102, 103]. Major growth-related processes controlled by TOR in yeast and multicellular organisms are ribosome biogenesis and translation [104]. An earlier study suggested that TOR controls the biosynthesis of ribosomal proteins and translation elongation factors via S6 kinase activation in mammals [105]. A very recent study shows that Tor1 directly binds to the promoter of 35S ribosomal DNA (rDNA) [106]. Starvation or treatment with rapamycin causes dissociation of Tor1 from the rDNA and withdrawal from the nucleus. This study also suggests that Tor1 regulates 35S rRNA synthesis by phosphorylation of the Pol I machinery [107] or by condensation of the rDNA repeats [108].

Reducing the TOR signaling activity extends life span in several model organisms [30, 93–98]. In yeast, treating cells with rapamycin, deleting *TOR1* or the downstream effectors/transcriptional targets involved in the utilization of alternative N sources, increases both replicative and chronological life span (Fig. 1a, b) [30, 97]. In addition, inhibiting the enzyme glutamine synthetase, which reduces cellular glutamine levels and TOR signaling [109], increases both chronological and replicative life span [30, 97]. Earlier studies implicated other N source-regulated downstream effectors of TOR, in particular the Rtg2 protein [110, 111] in modulation of yeast replicative and chronological life span (Fig. 1a, b) [53, 112, 113]. Supporting the role of amino acids and TOR in aging, removal of the preferred amino acids asparagine and glutamate from the culturing media prolongs chronological life span in yeast [97]. Furthermore, reduced concentrations of methionine in the media have been reported to extend yeast replicative life span, whereas increased methionine concentrations diminishes it [114]. Positive effects on life span through methionine reduction or overall protein dietary restriction in rodents has also been shown, although the maximal life span extension by amino acid/protein restriction is less than that of CR [115].

In worms, partial reduction in TOR function by RNAi treatments or mutations in CeTor or a conserved TOR-interacting protein, Raptor, proposed to relay nutrient signals [116], significantly extend worm life span (Fig. 2) [93, 95, 96]. In addition, interference with TOR function causes enlargement of the intestinal lumen and fat accumulation, developmental arrest at the L3 larval stage, and reduced fertility (decreased brood size), with the severity of this phenotype depending on the degree of TOR function elimination [93, 95, 96]. Overexpression of the fly/mammalian upstream negative regulators of TOR, Tsc1 and Tsc2 (tuberous sclerosis complex), as well as dominant-negative mutations of dTor or the fly/mammalian downstream effector of TOR, dS6K, extend life span in flies (Fig. 2) [94, 98]. In addition, overexpression of Tsc2 partially rescues the reduction of life span caused by increased concentrations of yeast extract in the fly diet [94].

The TOR and the insulin/PI3K pathways are interconnected in higher eukaryotes (Fig. 2) [95, 98, 117]. In worms, DAF-16 inhibits transcription of the TOR interacting partner - Raptor (DAF-15) [95]. In mammals and flies, TOR functions downstream of the insulin/PI3K pathway and cooperates in the regulation of cell growth and proliferation in response to growth factors, hormones and cytokines [98, 100]. Insulin/PI3K signaling to TOR is mediated through PKB, which in response to insulin/growth factors

alleviates inhibition of TOR by the negative regulatory factors Tsc1 and Tsc2 (Fig. 2) [98, 100]. Amino acid availability is signaled to TOR through the Tsc1-Tsc2 complex but is independent of PI3K [98]. On the other hand, it is also reported that TOR is necessary for the phosphorylation and activation of PKB/Akt, thus placing TOR upstream of PKB/Akt [117]. In addition, several studies in flies suggest that TOR signaling also has systemic effects – local amino acid/Tsc/TOR signaling in the larval fat body modulates organismal cell growth [118]. In addition to extracellular cues, TOR signaling may be regulated by intracellular amino acid and ATP or AMP concentrations [98, 119]. The cellular AMP/ATP ratio, sensed by the 5'-AMP-activated protein kinase (AMPK) [120, 121], acts upstream of TOR through regulation of the phosphorylation status of Tsc2 in mammals (Fig. 2) [98, 122, 123]. AMP-activated kinase has a major role in coupling metabolism to energy status in the cell; it down-regulates anabolic processes, such as fatty acid and cholesterol biosyntheses, and up-regulates catabolic processes, such as fatty acid oxidation in mammals [121, 124]. Snf1, the AMPK homologue in yeast, is required for transcription of genes involved in the metabolism of alternative carbon sources, respiration, gluconeogenesis, peroxisome biogenesis, glycogen storage, thermo-tolerance and sporulation [120]. Most importantly, subunits of the Snf1 kinase complex, and the AMP-activated kinase homologue in worm, AAK-2, are also shown to regulate life span [46, 125]. Furthermore, several studies have indicated that CR enhanced the phosphorylation and activation of AMPK in rat liver and heart [126, 127]. However, in a study in young adult mice, no significant changes in AMPK activity were observed after 4 months of CR [128].

It is currently unclear whether the TOR signaling pathway and CR function by similar mechanisms. Although recent studies in yeast suggest that reduction of TOR signaling and CR may function in the same pathway to regulate longevity [30], further studies are anticipated to validate this model both in yeast and in higher eukaryotes.

## Sirtuins

The life span-extending effect of CR observed in multiple model organisms is linked to a conserved sirtuin family [5, 48, 129–133]. The founding member, silent information regulator 2, Sir2, was first characterized in yeast where it regulates chromatin silencing at the mating type loci, telomeres and the rDNA repeats [134–136]. Sir2 exhibits an NAD-dependent histone deacetylase activity that is conserved among

the Sir2-family members [137–139]. In yeast, CR requires NAD and Sir2 [5, 48]. The longevity regulating activity of Sir2 involves suppressing the recombination among the rDNA repeats thereby decreasing the levels of toxic extrachromosomal rDNA circles (ERCs) (Fig. 1a) [87, 140]. Yeast Sir2 may also increase the fitness of newborn cells by asymmetric partitioning of the oxidatively damaged proteins to mother cells [141]. In yeast, overexpressing Sir2 extends the replicative life span by 30% [29]. Increasing the dosage of sirtuin in worms (*sir-2.1*) [142] and flies (*dSir2*) also extends life span [130]. Although it is still unknown whether the Sirtuins play an important role in mammalian aging, studies reveal that the targets of mammalian Sir2 proteins are involved in regulating cell survival under stress through controlling the acetylation status of both the tumor suppressor gene p53 [143, 144] and the FOXO transcription factors [145–147].

Sir2 proteins and their activation by CR represent a potential commonality in longevity pathways of multiple model organisms (Figs 1 and 2). Two models for Sir2 protein activation have been proposed. In yeast, CR induces a shunting of carbon metabolism from fermentation to the mitochondrial tricarboxylic acid (TCA) cycle [48]. The concomitant increase in respiration is necessary and sufficient for the activation of Sir2-mediated silencing and life span extension [48]. The fact that respiration produces NAD from NADH as well as the finding that NADH can function as a competitive inhibitor of Sir2 activity [50], reinforce the idea that an increase in the NAD/NADH ratio activates Sir2 during CR. In addition, genetic manipulations that cause a decrease in NADH levels are shown to increase Sir2 activity and extend life span [48, 50]. In mice, levels of the endothelial nitric oxide synthase (eNOS) increase during CR, mediating an increase in respiration and mitochondrial biogenesis [131]. Concomitant with an increase in eNOS concentration, enhanced expression of the mammalian Sir2 ortholog SIRT1 was observed, thus reinforcing the link between respiration and Sir2 activation [131]. This model, however, is challenged by two recent studies. One study suggests that CR does not require functional mitochondria to extend life span [148]. It is noteworthy that this study focuses on a more severe CR condition, 0.05% glucose, rather than a moderate CR condition, 0.5% glucose. It is very likely that 0.5% and 0.05% glucose are engaged in different pathways to extend life span and the discrepancies are due to different CR conditions and different strain backgrounds [149, 150]. Discrepancies between the concentrations required for *in vivo* and *in vitro* Sir2 inhibition by NADH have been reported. Another study suggests that the reported *in vivo*

NADH levels (~0.9 mM) are not sufficient to inhibit Sir2 activity since the  $IC_{50}$  of NADH for Sir2 *in vitro* is ~10 mM under their assay conditions [151]. It is very possible that intracellular compartmentalization of NAD and NADH and specific protein-protein interactions create local high NAD/NADH ratios, thereby activating Sir2 *in vivo*. In fact, it was suggested that the affinity/sensitivity of Sir2 toward its substrates and inhibitors varied when Sir2 was in complex with different interacting partners [152]. Alternatively, but not mutually exclusive, Sir2 activity is also regulated by the concentration of the non-competitive inhibitor nicotinamide, NAM, a byproduct of NAD degradation in the Sir2-mediated deacetylation reaction [153]. It is therefore suggested that CR extends life span by decreasing the level of NAM [51]. This model is further supported by the evidence that overexpressing nicotinamidase, Pnc1, increases yeast life span and suppresses the inhibitory effect of NAM on Sir2 deacetylase activity *in vivo* and *in vitro* [51, 154].

The focus on the link between CR and Sirtuin protein activity may have overshadowed the possibility that alternative targets of CR, as well as Sir2-independent pathways may play prominent roles in longevity regulation. In yeast, a Sir2-independent CR pathway has recently been described [60]. In this model, CR appears to extend life span in certain *sir2Δ* mutants when a second gene, *FOBI*, is deleted [60]. Deletion of the *FOBI* gene, which encodes an rDNA-specific replication fork block protein, decreases rDNA recombination thereby rescuing the short life span of the *sir2Δ* mutants to a wild-type level [155]. A *fob1Δ* mutation has been routinely introduced into the *sir2Δ* mutants to help reveal the true requirement of Sir2 in CR [48, 50, 52, 60]. Although two Sir2 family members, Hst2 and Hst1 have been suggested to play a role in this Sir2-independent pathway under 0.5% glucose CR [52], certain CR conditions such as 0.05% glucose appear to be totally independent of the Sir2 family [156, 157]. Therefore, different CR conditions (pathways) are likely to activate different, yet sometimes overlapping, downstream targets to extend life span. Further studies are required to elucidate the components and interactions of these multiple pathways of CR.

Recent studies have suggested the possible involvement of mammalian Sir2 homologs in longevity regulation, CR, stress response and cell survival. In agreement with a role for mammalian sirtuins in CR, several groups have demonstrated up-regulation of the expression of the closest mammalian Sir2 homolog, SIRT1, in brain, liver, muscle, kidney and adipose tissue upon CR or up to 24-h fasting in rodents [41, 131, 158–160]. Additionally, SIRT1 is required for the increase in physical activity upon CR in mice [132].

Both human and mouse SIRT1 have been shown to function as NAD-dependent p53 deacetylases [143, 144]. Deacetylation of p53 via SIRT1 promotes cell survival under stress [143, 144, 161] and may play a role in aging [162] and tumorigenesis [161]. Significantly, several SIRT1-interacting proteins have been identified. Mammalian SIRT1 attenuates Bax-mediated apoptosis by deacetylating the DNA repair factor Ku70 [41]. This study suggests CR could extend life span by inducing SIRT1 expression and by promoting the long-term survival of irreplaceable post-mitotic cells. Activating SIRT1 has also been shown to prevent neurodegeneration. Increasing NAD concentrations in the culture media or overexpressing an NAD biosynthetic enzyme nicotinamide mononucleotide adenylyl transferase 1 (Nmnat1) protects neuronal cells from axonal degeneration via activating the SIRT1 proteins [163]. Furthermore, increased protein levels and deacetylation activity of SIRT1 in mouse brain neurons during CR leads to a substantial decrease in  $\beta$ -amyloid content, and this reduction in Alzheimer disease-type amyloid neuropathology is due to SIRT1-mediated inhibition of the Ser/Thr Rho kinase ROCK1 expression and selective elevation of  $\alpha$ -secretase activity [159]. Although many studies have demonstrated that SIRT1 protected cells from apoptosis (Fig. 2), one study reported that activation of SIRT1 by resveratrol sensitizes mammalian cells to TNF $\alpha$ -induced apoptosis, and this elevated sensitivity is mediated by SIRT1 deacetylation of the p65 (RelA) subunit of NF- $\kappa$ B leading to inhibition of NF- $\kappa$ B-regulated anti-apoptotic gene expression [164]. It is noteworthy that histone deacetylases class I and II can also inhibit TNF $\alpha$ -induced NF- $\kappa$ B-mediated transcription [164]. Interestingly, the SIRT1-deficient mouse embryonic fibroblasts display an enhanced proliferative capacity upon chronic sublethal oxidative stress and impaired up-regulation of p19<sup>ARF</sup> and p53 protein levels, suggesting the SIRT1 effect on overall cellular p53 level and function is influenced by both the cell type and the strength of the applied stress stimuli [31].

Another group of studies directly linked SIRT1 to the longevity regulating insulin/IGF-1 pathway that contributes to the regulation of life span in worms, flies and mice [7]. One downstream target of the insulin/IGF-1 pathway in worms is the forkhead transcription factor DAF-16 [7], a homolog of the mammalian FOXO family of forkhead transcription factors, Foxo1 (FKHL), Foxo3 (FKHRL1), and Foxo4 (AFX), which transmit insulin signaling downstream of Akt kinase [80, 81]. It has been shown that Sir2-induced life span extension in worms requires functional DAF-16 [142], suggesting SIRT1 may also interact with the FOXO family in mammalian cells. Indeed, SIRT1 is able to

interact with Foxo1 and Foxo3 and prevents FOXO-induced cell death in a deacetylase-dependent manner [145–147]. SIRT1 also increases the ability of FOXO to activate antioxidant gene expression [145–147]. SIRT1 may therefore increase longevity by preventing apoptosis and increasing stress resistance in a FOXO-dependent manner (Fig. 2). Furthermore, it has been suggested that SIRT1-mediated deacetylation of nuclear FOXO1 upon oxidative or acute metabolic stress can temporarily promote pancreatic  $\beta$ -cell survival and function by up-regulating FOXO-1-dependent transcription, but ultimately leads to FOXO1 degradation and premature senescence of  $\beta$ -cells [165]. Significantly, starvation induces SIRT1 expression in mammalian cells, and this SIRT1 up-regulation is in part dependent on FOXO3 activation and its interaction with p53 upon nutrient withdrawal [158]. Thus, SIRT1, FOXO transcription factors and p53 comprise a homeostatic network regulating stress resistance, cell survival and organismal life span.

Finally, the Sir2 family may extend life span by regulating metabolic pathways [12, 166]. The Sir2 family has recently been connected to acetyl-CoA synthesis through deacetylation and activation of acetyl-CoA synthetase (ACS) [167–170] in multiple species. Decreasing SIRT1 activity in  $\beta$ -cell lines leads to increased UCP2 (an uncoupling protein) expression and decreased insulin secretion, creating a state that mimics food deprivation [171]. Furthermore,  $\beta$ -cell-specific SIRT1 overexpression in mice leads to improved glucose tolerance and enhanced insulin secretion in response to high glucose challenge [172]. On the other hand, the mitochondrial protein SIRT4 represses insulin secretion by ADP-ribosylation and inhibition of glutamate dehydrogenase (GDH) enzyme activity [173]. CR relieves GDH inhibition and boosts the amino acid-stimulated insulin secretion [173]. In addition, SIRT1 appears to modulate glucose metabolism through activation of PGC1- $\alpha$  (PPAR- $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ , coactivator 1- $\alpha$ ), leading to an increase in gluconeogenesis and a decrease in glycolysis in liver cells after fasting [160]. Adipose tissues in multicellular eukaryotes have an important role in longevity regulation. In support of this notion, mice lacking the insulin receptor, specifically in white adipose tissue, showed reduced fat mass and extended longevity [78]. In addition to increasing SIRT1 protein levels [131], CR up-regulates expression of SIRT3, a mammalian sirtuin important for mitochondrial function in both white and brown adipose tissues in mice [174]. SIRT1 has been demonstrated to promote fat mobilization in white adipose tissue by repressing the adipogenic nuclear hormone receptor PPAR- $\gamma$  [175]. Although fat mass has been proposed to be a key factor in



regulating longevity [12], it has also been shown that reduced adiposity is not required for CR life extension [176]. In summary, by creating system-wide changes in health, sirtuins may act to extend life span by increasing the global metabolic fitness of the organism. CR and SIRT1 expression lower body fat percentage, increase insulin sensitivity and stress resistance. We await future studies to link these cellular changes to whole organism activity and fitness.

### Metabolic fitness mediated by mitochondrial and stress signaling

Mitochondria are the primary site of cellular energy (ATP) generation, as well as the crossing point between catabolic and anabolic processes [177–180]. Recently, it has been shown that CR promotes mitochondrial biogenesis and respiration in mice through up-regulating the expression of eNOS and subsequent increase in NO and cyclic-GMP [131]. CR also up-regulates respiration in yeast [48]. Replicative life span extension by reduction of the glucose concentration from 2% to 0.5% requires an intact electron transport chain [48]. Genetic manipulations shifting metabolism from fermentation to respiration, such as Hap4 overexpression, also prolong yeast replicative life span [48]. Hap4 is the regulatory subunit in the Hap2/3/4/5 complex, which activates transcription of genes involved in the electron transport chain, TCA cycle and heme biosynthesis [181, 182]. Several lines of evidence suggest that mitochondrial function is also important for chronological life span in yeast. Inhibition of oxidative phosphorylation by treatment with antimycin A decreases chronological life span [113]. Conversely, increasing the electron flow through the respiratory chain by treatment with 2,4-dinitrophenol (uncouples electron transport from ATP production), increases chronological life span. In addition, inhibiting TOR by rapamycin treatment up-regulates respiration by inducing the expression of genes encoding TCA cycle enzymes and proteins involved in oxidative phosphorylation [86, 88].

Inhibition of TOR evokes the retrograde response – a signaling pathway responding to the functional state of mitochondria through changes in the nuclear gene expression [110, 183]. Through up-regulating the expression of anaplerotic and TCA cycle enzymes, and peroxisomal proteins, the TOR/retrograde response provides a way for the yeast cell to compensate for decreased mitochondrial function and maintain adequate biosynthetic capacity [110, 183, 184]. Key components of the TOR/retrograde response are the basic helix-loop-helix/leucine zipper transcription

factors Rtg1 and Rtg3, and their upstream activator Rtg2, a cytoplasmic protein with an Hsp70 type ATP-binding domain homologous to that of bacterial phosphatases hydrolyzing ppGpp (guanosine 3', 5'-bis-pyrophosphate) [110, 183]. The retrograde response has been implicated as one of the determinants of longevity in yeast (Fig. 1a) [53, 112, 185]. However, its precise role for yeast longevity is somewhat controversial – on one hand functional retrograde response is required for increased replicative life span in respiratory-deficient cells known as petites; deletion of *RTG2* eliminates the extension of life span in petites. On the other hand, disruption of *RTG*-signaling through deletion of *RTG3* also leads to an increased replicative life span [53, 112, 185]. One explanation put forward is that, although the induction of the retrograde response compensates for age-associated mitochondrial dysfunction, it also progressively monopolizes the activity of Rtg2 protein, and thus Rtg2 is not available to fulfill its other functions, such as the repression of genome instability at the rDNA repeats [112, 186]. It is currently unclear whether the TOR signaling pathway or any other CR model functions through the retrograde response to extend life span.

Although many studies support the importance of increased mitochondrial function for longevity, several studies suggest that a decrease in mitochondrial function, in particular a decrease in respiration, extends life span [89, 90, 187]. Specifically, partial inhibition of mitochondrial respiration during worm larval development extends adult life span in a DAF-16-independent manner [89, 90, 187]. It is important to note that these mitochondrially compromised long-lived worms exhibit developmental delays as well as growth and behavioral alterations. RNAi disruption of respiratory electron transport and ATP synthesis genes increases life span only if applied during worm development, indicating that the timing of the inhibition of the mitochondrial respiration is crucial for life span extension [89, 187]. Furthermore, the long-lived *clock-1* (*clk-1*) mutant worms are deficient in ubiquinone synthesis and also show developmental and behavioral changes [188–190]. Heterozygosity for the mouse ortholog of *clk-1* extends life span, and it has been speculated that this life span extension may be due to a decrease in ROS production as a result of slowing down the activity of the mitochondrial electron transport chain, ultimately leading to a reduction in DNA damage, as seen in *mclk-1*<sup>+/-</sup> liver cells [191]. Interestingly, combining the *C. elegans* dietary restriction mimic *eat-2* with the *clk-1* mutation does not significantly increase the long life span of either of the two single mutations [192]. It has also been reported that a more severe CR condition in

yeast does not require functional mitochondria to extend replicative life span [148]. Thus, the precise role of mitochondria in longevity regulation is still controversial but studies so far have unequivocally shown that this organelle has key importance in determining life span.

Mitochondria are the major source of ROS, and have been implicated in controlling cell death [179, 180, 193, 194]. An early theory of aging proposes that aging is caused by deleterious actions of ROS on cellular macromolecules, thus mitochondria, and in particular respiration, have a central importance in the aging process [195]. This model is supported by many studies, for example, transgenic mice overexpressing human catalase (detoxifies hydrogen peroxide) localized to the mitochondria show longer life span and decreased severity of age-associated diseases, such as arteriosclerosis, cardiomyopathy, cataracts [196]. Furthermore, joint overexpression of superoxide dismutase and catalase extends life span in yeast (chronological life span) and mice [37, 196]. Additionally, mice carrying deletions in the P66<sup>shc</sup> redox-sensing protein are resistant to stress-induced cell death and also exhibit longer life span [197, 198]. Paradoxically, CR appears to up-regulate mitochondrial biogenesis and respiration [48, 131]. While debate over the validity of the free radical theory of aging still rages, it becomes apparent that CR can protect cells from oxidative damage, but the importance of this role of CR in aging retardation is still unresolved [20, 195, 199–201]. CR decreases the rate of mitochondrial ROS production in yeast [113] and in rats [115, 200]. CR appears to decrease the amount of free radical leak specifically at complex I of the electron transport chain and, therefore, reduces mitochondrial DNA damage [115, 200]. Furthermore, CR diminishes oxidative damage by decreasing the degree of fatty acid unsaturation in cellular membranes – it promotes a shift in the membrane composition from highly unsaturated to less unsaturated fatty acids [200, 202]. Attenuating signaling through nutrient responsive pathways is associated with increased resistance to oxidative stress in model systems [73]. In yeast, the long-lived *sch9Δ* mutants require mitochondrial superoxide dismutase (Sod2) for life span extension (Fig. 1b) [37]. Additionally, long-lived low PKA activity mutants exhibit increased stress resistance mediated by the stress-sensing transcription factors Msn2 and Msn4 [5, 6, 37]. Msn2/Msn4 play a key role in regulating the expression of stress response element (STRE)-controlled genes [203]. STRE-controlled genes are induced upon heat shock, osmotic stress, carbon-source deprivation, ethanol or sorbic acid treatment [203]. It is noteworthy that regulation of the expression of these genes is at the cross point of

several signal transduction pathways, including TOR, PKA, Sch9, and Snf1 pathways [67, 203–205]. Interestingly, while Msn2/4 are required for chronological life span extension induced by low PKA activity mutations [6, 37] and the *gln3Δ* mutation [97], it is dispensable for replicative life span extension in the low PKA activity mutants [5, 206]. Therefore, if stress signaling is indeed the key regulator of longevity, the corresponding pathways that affect replicative life span in yeast are yet to be identified.

To partly reconcile the role of CR in promoting mitochondrial biogenesis and respiration, and at the same time decreasing oxidative damage, it has been suggested that glycolysis is a greater source of reactive intermediates capable of modifying cellular macromolecules [207]. Another popular hypothesis is the “Hormesis” theory: CR acts as a “nutritional stress” and mobilizes various defense mechanisms to elicit a well-coordinated multi-level protection [20, 21, 202]. Thus, low-intensity stress, induced by CR, can evoke metabolic, transcriptional, translational and other cellular and organismal changes that lead to a stronger resistance to various forms of stress [20, 21, 202]. In support of this notion, reducing signaling through the nutrient responsive pathways leads to increased resistance to multiple stresses. While the “Hormesis” hypothesis has not won final approval, it conveniently unifies various findings and previous hypotheses under one umbrella [20, 21]. Studies discussed herein also suggest that CR may increase life span by increasing or maintaining mitochondrial activity, leading to enhanced metabolic fitness, since the activity of many metabolic enzymes declines with age. CR not only decreases the level of ROS but also prevents specific age-induced damage to these metabolic enzymes [113, 199, 208]. Future studies on identification and characterization of the downstream mediator/targets of CR and the longevity regulating nutrient-sensing pathways will help to elucidate the mechanisms of longevity regulation and possibly the mechanisms of age-associated diseases in humans.

### Summary and outlook

In conclusion, longevity is determined by both genetic and environmental factors. The nutrient, energy and hormonally regulated TOR, AMPK, insulin/PI3K, AKT/Sch9 and PKA (in yeast) pathways are among the prominent genetic factors modulating longevity. Reducing signaling through these pathways increases life span in multiple species. In addition, it is suggested that insulin/PI3K and TOR signaling may also affect life span through systemic effects in multicellular organisms. CR is the most powerful environmental

intervention that promotes healthy long life in almost all organisms. Extension of life span by CR is mediated by both sirtuin-dependent and sirtuin-independent mechanisms. Activation of the highly conserved NAD-dependent deacetylases of the sirtuin family increases life span in yeast, worms and flies. Activating sirtuins and reducing signaling through nutrient sensing pathways extends life span by impinging on a variety of cellular processes such as metabolic alterations and acquisition of stress resistance. Owing to the remarkable evolutionary conservation in the longevity regulating proteins and signaling pathways, studies of longevity regulation in model organisms have contributed and will continue to provide a wealth of information relevant to human aging and age-associated diseases.

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